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(54) Title: COMPOSITIONS AND METHODS FOR THE PREVENTION OR TREATMENT OF CANCER AND BONE LOSS ASSOCIATED WITH CANCER

(57) Abstract: The present invention relates to compositions and methods for the prevention and/or treatment of bone loss associated with cancer. More particularly, the invention relates to OPG compositions and methods for the prevention and/or treatment of bone loss comprising said compositions. The invention also relates to the use of OPG compositions for the treatment of multiple myeloma.

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COMPOSITIONS AND METHODS FOR THE PREVENTION OR
TREATMENT OF CANCER AND BONE LOSS ASSOCIATED WITH
CANCER

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Field of the Invention

The present invention relates to compositions and methods for the prevention and/or treatment of bone loss associated with cancer. More particularly, the invention relates to compositions comprising OPG and methods for the prevention and/or treatment of bone loss comprising said compositions. The invention also relates to the use of OPG compositions for the treatment of multiple myeloma.

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Background of the Invention

Many cancers can become established in tissues and organs which are far removed from the original site of tumor growth. Such cancers, termed metastatic cancers, can cause widespread complications that are often fatal. The skeleton is a common site for the spread of solid tumors, exceeded in frequency by only the liver and the lung. As a result of invasion by cancer cells, osteoclasts, the primary cells in bone that promote bone resorption, become hyperactivated and begin to break down bone at an accelerated rate. Osteoclasts are activated by substances such as parathyroid hormone-related peptide (PTHrP) and interleukin-1 (IL-1), both of which are increased in the bone microenvironment and are also produced by tumor cells. Patients with bone cancer frequently develop lytic bone lesions as a result of increased osteoclast activity. This condition is referred to as osteolytic bone metastasis. Bone lysis can lead to pathologic fractures, spinal collapse,

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hypercalcemic events and bone pain and is a major cause of mortality and morbidity. Alternatively, as in prostate cancer bone metastases, increased osteoclastic bone destruction is accompanied by increased but

5 disorganized bone formation (Kylmaelae et al. Brit. J. Cancer 71, 1061-1064 (1995)). The original bone is removed, and replaced by woven unstructured bone so that the architectural integrity of the bone is lost. This also results in bone pain and other morbidities.

10 In addition osteoclast activity may increase the propensity of cancer cells to metastasize to bone and then to grow in that environment. Osteoclasts have been shown to release cytokines such as IL-6 which is a growth factor for some hematologic tumor cells such as
15 multiple myeloma cells (O'Keefe et al. Lab. Invest. 76, 457-465 (1997)). In addition, osteoclasts have been shown to release growth factors from bone matrix during bone resorption. These include fibroblast growth factors and transforming growth factor β which are
20 known to promote growth of many solid tumors. In this way osteoclastic activity could create a fertile environment for metastatic seeding within bone, and as the tumor cells begin to grow and promote bone resorption, cause release of growth factors from the
25 bone to sustain tumor expansion.

Currently available cancer therapy agents can reduce or inhibit tumor growth but have little effect on underlying lytic bone disease. It has been reported that some chemotherapeutic regimens actually contribute
30 to bone loss associated with hematological malignancies such as multiple myeloma and Hodgkin's disease and in the case of gonadotrophin releasing hormone receptor agonists. In addition, once cancer has spread to the bone, it becomes more difficult to treat using current
35 regimens. It is therefore desirable to be able to

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prevent the development of bone metastases and to treat bone metastases to prevent bone loss at an early stage.

Bone anti-resorptive agents inhibit the number and/or activity of osteoclasts and reduce the rate at which bone is broken down. Such agents may be useful in preventing and/or treating bone resorption associated with bone cancer. It has been reported that bisphosphonates such as risedronate, ibandronate and pamidronate, which are anti-resorptive compounds, can reduce the severity of skeletal events (e.g., pathological fractures, spinal collapse, radiation of or surgery on bone) in mouse tumor models and in patients suffering from breast cancer and multiple myeloma and other tumor bone metastases. In addition bisphosphonates have been reported to reduce bone pain and other skeletal events in prostate cancer bone metastases. However, bisphosphonates have been shown to have limited efficacy with only a modest reduction in skeletal events even when given in high doses by infusion. When taken orally, bisphosphonates have reduced efficacy and can cause gastrointestinal irritation (e.g., heartburn, dyspepsia and nausea) and in some cases esophageal ulcers if not administered properly.

Osteoprotegerin (OPG) has been described in PCT Publication No. WO97/23614 and found to negatively regulate formation of osteoclasts in vitro and in vivo. OPG dramatically increased the bone density in transgenic mice expressing the OPG polypeptide and reduced the extent of bone loss when administered to ovariectomized rats. An analysis of OPG activity in in vitro osteoclast formation revealed that OPG blocks the differentiation of osteoclasts from monocyte/macrophage precursors. OPG appears to have specificity in regulating the extent of osteoclast formation. OPG is a potent factor in blocking bone resorption and may be

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used in the prevention and treatment of loss of bone mass. In vitro and in vivo activity of inhibiting osteoclast formation and blocking loss of bone was also observed in fusion proteins comprising OPG and an Fc domain.

Consequently, it is an object of the invention to provide alternative methods and compositions for the treatment of bone loss associated with cancer that overcome many of the problems associated with current therapy.

It is a further object of the invention to provide alternative methods and compositions for the prevention of bone loss associated with cancer by prophylactic treatment to decrease the incidence of bone metastasis and/or to delay the onset of bone metastasis.

It is a further object of the invention to provide alternative methods and compositions to prevent and/or treat multiple myeloma.

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Summary of the Invention

The invention provides for a method of preventing or treating a lytic bone disease in a mammal comprising administering a therapeutically effective amount of an OPG polypeptide. Lytic bone disease is commonly observed in a mammal suffering from cancer which has metastasized to bone. Examples of such cancers include breast, prostate, thyroid, kidney, lung, esophageal, rectal, bladder, and cervical cancers as well as cancer of the gastrointestinal tract. Also included are certain hematological malignancies, such as multiple myeloma, leukemia and lymphomas, such as Hodgkin's Disease. Also included is metastatic bone disease that increases both bone resorption and bone formation resulting in osteosclerotic bone metastases

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or mixed lytic and osteosclerotic metastases which are associated with bone pain and the loss of the structural integrity of bone as in tumors such as prostate cancer.

- 5 The invention also provides for a method of preventing metastasis of cancer to bone comprising administering a therapeutically effective amount of an OPG polypeptide.

- The invention also provides for a method of
10 preventing or treating a metastatic bone disease in a mammal comprising administering a therapeutically effective amount of an OPG polypeptide in combination with a cancer therapy agent. The cancer therapy agent may be any agent which is used to treat tumor growth
15 including radiation therapy and chemotherapeutic drugs. Examples of such agents include anthracyclines, taxol, tamoxifen, antibodies, such as anti-Her2 or anti-CD20 antibodies, and receptor agonists and antagonists, such as luteinizing hormone-releasing hormone (LHRH)
20 antagonists. OPG polypeptide compositions may be administered prior to, concurrent with, or subsequent to administration of a cancer therapy agent.

- The invention also provides for a method of treating multiple myeloma comprising administering a
25 therapeutically effective amount of an OPG polypeptide.

- OPG polypeptides of the invention encompass those polypeptides which have the activity of inhibiting bone resorption and may be used to prevent and/or treat loss of bone mass or prevent
30 osteosclerotic bone metastasis (replacement of structurally sound bone with disorganized structurally deficient bone). In preferred embodiments, OPG polypeptides are fusion proteins comprising OPG and a heterologous peptide or protein. Such fusion proteins
35 can exhibit increased circulating half-lives and slower clearance times, thereby providing a more sustained

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anti-resorptive activity and less frequent administration. In one aspect, the heterologous protein is an immunoglobulin Fc region, or a variant, fragment or derivative thereof.

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Description of the Figures

Figure 1 shows the amino acid sequence of the hinge, CH2 and CH3 regions of human IgG1.

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Figure 2 shows the amino acid sequence of human OPG [1-401].

Figure 3 shows the amino acid sequence of OPG[22-194]-Fc.

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Figure 4 shows the amino acid sequence of OPG[22-201]-Fc.

Figure 5 shows the amino acid sequence of OPG[22-194]-FcΔC.

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Figure 6 shows the amino acid sequence of OPG[22-201]-FcΔC.

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Figure 7 shows the amino acid sequence of OPG[22-194]-FcG₁₀.

Figure 8 shows and amino acid sequence of metFcΔC-OPG[22-194].

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Figure 9 shows prevention of osteolytic bone destruction in C26-DCT and MDA-MB-231 models of tumor metastasis to bone. Both cells types produce localized bone destruction (yellow arrows) following inoculation

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- directly into the left ventricle of mice. Panels on the left side show that radiographic lesions are evident 10 days after administration of C26-DCT cells and 28 days after administration of MDA-MB-231 cells.
- 5 Panels on the right side show prevention of lesion formation after treatment with metFcΔC-OPG[22-194] (25mg/kg) every 3 days for 10 days (for C26-DCT mice) or 3 times/week for 4 weeks (for MDA-MB-231 mice).
- 10 Figure 10 shows a metFcΔC-OPG [22-194] dose dependent reduction in the number of radiographically evident osteolytic foci in mice inoculated with C26-DCT cells.
- 15 Figures 11A and 11B show prevention and reversal of hypercalcemia associated with malignancy in a mouse C26-DCT tumor model. In the prevention study, met FcΔC-OPG[22-194] was given by daily subcutaneous injection. In the reversal study, met FcΔC-OPG[22-194]
- 20 was given by a single intravenous injection. Mean blood calcium levels \pm SEM are reported.

Detailed Description of the Invention

- 25 The present invention provides for compositions and methods for the prevention and treatment of bone loss associated with cancer. The present invention also provides for methods for the prevention and treatment of cancer using an anti-
- 30 resorptive bone agent. Preferred compositions and methods of the invention include OPG and OPG fusion polypeptides. More particularly, the present invention relates to the use of OPG fusion protein compositions for the prevention and/or treatment of cancer or for

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the prevention and/or treatment of bone loss associated with cancer.

Unexpectedly, it has been observed that fusion of an Fc region to a truncated OPG polypeptide demonstrates advantages which are not seen with unfused truncated OPG polypeptides or with full-length mature OPG. (wherein full-length mature OPG has 380 amino acids, such as from residues 22 to 401 inclusive, as shown in Figure 2 (SEQ ID NO: 2) It has been further observed that fusion of an Fc region at the carboxy terminus of an OPG polypeptide provides unexpected advantages compared to fusion of an Fc region at the amino terminus of an OPG polypeptide. Accordingly, OPG fusion proteins, and variants, fragments and derivatives thereof, as well as, related methods of use and preparation, are described in more detail below.

The term "OPG" or "OPG polypeptide" refers to a polypeptide comprising the amino acid sequence as set forth in Figure 2 (SEQ ID NO: 2) and related polypeptides described herein. Related polypeptides include allelic variants; splice variants; fragments; derivatives; substitution, deletion and insertion variants; fusion polypeptides; and non-human homologs. OPG polypeptides may be mature polypeptides, as defined herein, which may or may not have an amino terminal methionine residue, depending upon the method of preparation.

The term "OPG fusion protein" refers to an OPG protein, or OPG polypeptide which is joined to a heterologous peptide or polypeptide. The OPG fusion proteins of the invention may be prepared by any suitable means known in the art, such as by genetic or chemical fusion of OPG and heterologous peptide or polypeptide moieties. In an embodiment of the invention, the heterologous peptide or polypeptide is an Fc region of an immunoglobulin, preferably a human

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immunoglobulin. A heterologous peptide or protein may be joined either to the amino terminus or to the carboxy terminus of an OPG polypeptide.

The term "mature OPG polypeptide" or "mature OPG fusion polypeptide" refers to a polypeptide or a fusion polypeptide lacking a leader sequence and may also include other modifications such as proteolytic processing of the amino terminus (with or without a leader sequence) and/or the carboxy terminus, cleavage of a smaller polypeptide from a larger precursor, N-linked and/or O-linked glycosylation, and the like.

The term "Fc" refers to a molecule or sequence comprising the sequence of a non-antigen-binding portion of antibody, whether in monomeric or multimeric form. The original immunoglobulin source of an Fc is preferably of human origin and may be from any isotype, e.g., IgG, IgA, IgM, IgE or IgD. One method of preparation of an isolated Fc molecule involves digestion of an antibody with papain to separate antigen and non-antigen binding portions of the antibody. Another method of preparation of an isolated Fc molecules is production by recombinant DNA expression followed by purification of the Fc molecules so expressed. A full-length Fc consists of the following Ig heavy chain regions: C_H1, C_H2 and C_H3 wherein the C_H1 and C_H2 regions are typically connected by a flexible hinge region. In one embodiment, an Fc has the amino acid sequence of IgG, such as that shown in Figure 1. The terms "Fc protein", "Fc sequence", "Fc molecules", "Fc region" and "Fc portion" are taken to have the same meaning as "Fc".

The term "fragment" when used in association with Fc or OPG polypeptides, or with fusion polypeptides thereof, refers to a peptide or polypeptide that comprises less than the full length amino acid sequence of an Fc or OPG polypeptide. Such

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a fragment may arise, for example, from a truncation at the amino terminus, a truncation at the carboxy terminus, and/or an internal deletion of a residue(s) from the amino acid sequence. OPG or Fc fragments may
5 result from alternative RNA splicing or from *in vivo* protease activity.

The term "variant" when used in association with Fc or OPG polypeptides, or with fusion polypeptides thereof, refers to a polypeptide
10 comprising an amino acid sequence which contain one or more amino acid sequence substitutions, deletions, and/or additions as compared to native Fc or OPG polypeptide amino acid sequences. Variants may be naturally occurring or artificially constructed.
15 Variants of the invention may be prepared from the corresponding nucleic acid molecules encoding said variants, which have a DNA sequence that varies accordingly from the DNA sequences for native Fc or OPG polypeptides.

20 The term "derivative" when used in association with Fc or OPG polypeptides, or with fusion polypeptides thereof, refers to Fc or OPG polypeptide variants or fragments thereof, that have been chemically modified, as for example, by covalent
25 attachment of one or more polymers, including, but limited to, water soluble polymers, N-linked or O-linked carbohydrates, sugars, phosphates, and/or other such molecules. The derivatives are modified in a manner that is different from native Fc or OPG, either
30 in the type or location of the molecules attached to the polypeptide. Derivatives further includes deletion of one or more chemical groups naturally attached to an Fc or OPG polypeptide.

The term "fusion" refers to joining of
35 different peptide or protein segments wherein the joined ends of the peptide or protein segments may be

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directly adjacent to each other or may be separated by linker or spacer moieties such as amino acid residues or other linking groups. A fusion may be accomplished by genetic or chemical means using procedures available to one skilled in the art although the means of joining is not limited to those disclosed herein.

Polypeptides

The invention provides for OPG fusion polypeptides and compositions thereof, and more particularly provides for fusion polypeptides comprising OPG and Fc moieties. Fusions of an Fc region to an OPG polypeptide may be made at the amino terminus of OPG, that is, the carboxy terminus of an Fc region is fused to the amino terminus of OPG. These fusion proteins (and nucleic acids encoding same) are designated herein as FcOPG. It may also be desirable to fuse the carboxy terminus of OPG to the amino terminus of an Fc region. These fusion proteins (and nucleic acids encoding same) are designated herein as OPGFc.

An Fc, or a variant, fragment or derivative thereof, may be from an immunoglobulin (Ig) class. In one embodiment, an Fc is from the IgG class, such as IgG₁, IgG₂, IgG₃, and IgG₄. In another embodiment, an Fc is from IgG₁. An Fc may also comprise amino acid residues represented by a combination of any two or more of the Ig classes, such as residues from IgG₁ and IgG₂, or from IgG₁, IgG₂ and IgG₃, and so forth. In one embodiment, an Fc region of an OPG fusion protein has the sequence as set forth in Figure 1 (SEQ ID NO:___) comprising hinge C_H2 and C_H3 regions of human IgG1. (see Ellison et al., Nucleic Acids Res. 10, 4071-4079 (1982)).

In addition to naturally occurring variations in Fc regions, Fc variants, fragments and derivatives

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- may contain non-naturally occurring changes in Fc which are constructed by, for example, introducing substitutions, additions, insertions or deletions of residues or sequences in a native or naturally occurring Fc, or by modifying the Fc portion by chemical modification and the like. In general, Fc variants, fragments and derivatives are prepared such that the increased circulating half-life of Fc fusions to OPG is largely retained.
- Also provided by the invention are Fc and OPG variants with conservative amino acid substitutions. The term "conservative amino acid substitution" refers to a substitution of a native amino acid residue with a nonnative residue such that there is little or no effect on the polarity or charge of the amino acid residue at that position. For example, a conservative substitution results from the replacement of a non-polar residue in a polypeptide with any other non-polar residue. General rules for conservative amino acid substitutions are set forth in Table I.

Table I
Conservative Amino Acid Substitutions

Original Residues	Exemplary Substitutions	Preferred Substitutions
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln, His, Lys, Arg	Gln
Asp	Glu	Glu
Cys	Ser	Ser
Gln	Asn	Asn
Glu	Asp	Asp
Gly	Pro, Ala	Ala

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His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys	Arg, Gln, Asn	Arg
Met	Leu, Phe, Ile	Leu
Phe	Leu, Val, Ile, Ala, Tyr	Leu
Pro	Ala	Ala
Ser	Thr	Thr
Thr	Ser	Ser
Trp	Tyr, Phe	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Met, Leu, Phe, Ala, Norleucine	Leu

- Conservative amino acid substitutions also encompass non-naturally occurring amino acid residues which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics, and other reversed or inverted forms of amino acid moieties. Conservative modifications to the amino acid sequence (and the corresponding modifications to the encoding nucleotides) are expected to produce Fc and OPG molecules (and OPG fusion proteins) having functional and chemical characteristics similar to those of unmodified Fc, OPG and OPG fusion proteins.

- In addition to the substitutions set forth in Table I, any native residue in an Fc region or in an OPG polypeptide (or in an FcOPG fusion protein) may also be substituted with alanine, as has been previously described for "alanine scanning mutagenesis" (Cunningham et al. Science 244, 1081-1085 (1989)).

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Substantial modifications in the functional and/or chemical characteristics of an Fc or OPG polypeptide (and an OPG fusion protein) may be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the molecular backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues may be divided into groups based on common side chain properties:

- 1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
- 2) neutral hydrophilic: Cys, Ser, Thr;
- 3) acidic: Asp, Glu;
- 4) basic: Asn, Gln, His, Lys, Arg;
- 5) residues that influence chain orientation: Gly, Pro; and
- 6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class. Such substituted residues may be introduced into regions of an Fc or OPG molecule that are homologous with non-human Fc or OPG, or into the non-homologous regions of the molecule.

Cysteine residues in Fc molecules can be deleted or replaced with other amino acids to prevent formation of disulfide crosslinks. In particular, a cysteine residue at position 5 of Figure 1 (SEQ. ID. NO. 1) may be substituted with one or more amino acids, such as alanine or serine. Alternatively, the cysteine residue at position 5 could be deleted.

An Fc fragment may be prepared by deletion of one or more amino acids at any of positions 1, 2, 3, 4 and 5 as shown in Figure 1 (SEQ ID NO. 1). In one Fc

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molecule, FcΔC, the amino acid residues at positions 1-5 inclusive are deleted. Substitutions at these positions can also be made and are within the scope of this invention.

- 5 Fc variants may also be made which show reduced binding to Fc receptors which trigger effector functions such as antibody dependent cellular cytotoxicity (ADCC) and activation of complement. Such variants may include leucine at position 20 deleted or
10 substituted with a glutamine residue, glutamate at position 103 deleted or substituted with an alanine residue, and lysines at positions 105 and 107 deleted or substituted with alanine residues (following the numbering as set forth in Figure 1). One or more of
15 such substitutions are contemplated.

In one embodiment, Fc variants will exhibit stronger binding to the FcRn receptor ("salvage receptor") and a longer circulating half-life compared to native Fc. Examples of such variants include amino
20 acid substitutions at one or more of residues 33, 35-42, 59, 72, 75, 77, 95-98, 101, 172-174, 215 and 220-223 as shown in Figure 1 (SEQ ID NO: 1), wherein the substitution(s) confer tighter binding of an Fc variant to the FcRn receptor.

- 25 Other Fc variants include one or more tyrosine residues replaced with, for example, phenylalanine residues. In addition, other variant amino acid insertions, deletions and/or substitutions are also contemplated and are within the scope of the
30 present invention. Examples include Fc variants disclosed in WO96/32478 and WO97/34630 hereby incorporated by reference. Furthermore, alterations may be in the form of altered amino acids, such as peptidomimetics or D-amino acids.

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An Fc protein may be also linked to an OPG moiety of the OPG fusion polypeptide by spacer or linker moieties. Such spacers or linkers may be proteinaceous in that they comprise one or more amino acids or they may be chemical linkers. Such chemical linkers are well known in the art. Amino acid linker sequences can include but are not limited to:

- (a) ala-ala-ala;
- (b) ala-ala-ala-ala;
- 10 (c) ala-ala-ala-ala-ala;
- (d) gly-gly;
- (e) gly-gly-gly;
- (f) gly-gly-gly-gly-gly;
- (g) gly-gly-gly-gly-gly-gly-gly;
- 15 (h) gly-pro-gly;
- (i) gly-gly-pro-gly-gly;
- (j) val;
- (k) ser-gly-gly-gly-gly-gly-gly-gly-gly-gly;
- 20 (l) gly-gly-ser-gly-ser-gly-ala-gly-ser-gly-ser-gly-gly-gly-ser-gly-ser-gly-gly;
- (m) a chemical moiety; and
- (n) any combination of subparts (a) through (m).

25

OPG variants, fragments and derivatives are also provided by the invention and are generally as described hereinabove for Fc molecules, with the exception of the specific locations of the modified amino acid residues. OPG variants, fragments and derivatives are described in PCT WO97/23614 hereby incorporated by reference.

In a preferred embodiment, the OPG moiety of an OPG fusion protein is a carboxy-terminal truncated form of OPG. Carboxy terminal truncated forms of OPG have one or more amino acids from positions 186-401 as

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shown in Figure 2 deleted. For example, OPG truncations comprise the amino acid sequence 22-X wherein X is any residue from 185 to 400 inclusive. In another embodiment, OPG truncations comprise the amino acid sequence 22-X wherein X is any residue from 185 to 278 inclusive, or from 185 to 293 inclusive, or alternatively, from 194 to 278 inclusive, or from 194 to 293 inclusive. Fusion proteins comprising the OPG truncated polypeptides described herein encompass joining of the OPG and heterologous peptide or polypeptide moieties directly or through a spacer or linker molecule wherein the spacer or linker optionally comprises one or more amino acid residues. Variants and derivatives of the OPG truncated forms described herein are also encompassed by the invention.

Preferred fusion proteins of the invention include those wherein the OPG moiety comprises the amino acid sequence 22-X wherein X is any residue from positions 194 to 201 inclusive using the numbering as shown in Figure 2 (SEQ ID NO: 2). Examples of such fusion proteins include the following:

	OPG [22-194]-Fc	(Figure 3 and SEQ ID NO: 3)
	OPG [22-201]-Fc	(Figure 4 and SEQ ID NO: 4)
25	OPG [22-194]-FcΔC	(Figure 5 and SEQ ID NO: 5)
	OPG [22-201]-FcΔC	(Figure 6 and SEQ ID NO: 6)
	OPG [22-194]-FcG ₁₀	(Figure 7 and SEQ ID NO: 7)
	FcΔC-OPG [22-194]	(Figure 8 and SEQ ID NO: 8)

30 For the preferred polypeptides, the term "Fc" refers to the sequence of human IgG, shown in Figure 1, the term "FcΔC" refers to the sequence shown in Figure 1 (SEQ ID NO: 1) lacking amino acid residues 1-5 inclusive, and the term "FcG₁₀" refers to an Fc moiety

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lacking amino acid residues 1-9 inclusive, and having a ser-(gly)₈ linker.

Nucleic acid molecules

5 Nucleic acid molecules encoding OPG fusion proteins of the invention, or variants, fragments or derivatives thereof, are provided for by the invention. Nucleic acid molecules of the invention may be produced by recombinant DNA methodology known to one skilled in
10 the art. See, for example, Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory Press, Cold Springs Harbor, N.Y. (1989)), and Ausubel et al. (Current Protocols in Molecular Biology, Wiley and Sons, N.Y. (1994)), for descriptions
15 of mutagenesis techniques. Chemical synthesis using methods described by Engels et al. (Angew. Chem. Intl. Ed. 28, 716-734 (1989)), may also be used to prepare such variants. Other methods for preparing nucleic acid molecules known to the skilled artisan may also be
20 used.

In certain embodiments, nucleic acid molecules encode OPG fusion protein variants with conservative amino acid substitutions as defined hereinabove. For example, conservative amino acid
25 substitutions are made in an OPG and/or in an Fc moiety of a fusion protein. Also provided for are Fc or OPG variants comprising an addition and/or a deletion of one or more N-linked or O-linked glycosylation sites, or comprising Fc or OPG polypeptide fragments as
30 described above. It is understood that the nucleic acid molecules of the invention may encode any combination of Fc and/or OPG variants, fragments, and fusion polypeptides described herein.

In another embodiment, nucleic acids of the
35 invention contain codons which have been altered for optimal expression of an OPG fusion polypeptide in a

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given host cell. Particular codon alterations will depend upon the OPG fusion polypeptide(s) and host cell(s) selected for expression. Such "codon optimization" can be carried out by a variety of methods, for example, by selecting codons which are preferred for use in highly expressed genes in a given host cell. Computer algorithms which incorporate codon frequency tables such as "Ecohigh. Cod" for codon preference of highly expressed bacterial genes may be used and are provided by the University of Wisconsin Package Version 9.0, Genetics Computer Group, Madison, WI. Other useful codon frequency tables include "Celegans_high.cod", "Celegans_low.cod", "Drosophila_high.cod", "Human_high.cod", "Maize_high.cod", and "Yeast_high.cod".

Vectors and Host cells

A nucleic acid molecule encoding an OPG fusion polypeptide is inserted into an appropriate expression vector using standard ligation techniques. The vector is typically selected to be functional in the particular host cell employed (i.e., the vector is compatible with the host cell machinery such that amplification of the gene and/or expression of the gene can occur). A nucleic acid molecule encoding an OPG protein may be amplified/expressed in prokaryotic, yeast, insect (baculovirus systems) and/or eukaryotic host cells. Selection of the host cell will depend in part on whether an OPG protein is to be post-translationally modified (e.g, glycosylated and/or phosphorylated). If so, yeast, insect, or mammalian host cells are preferable.

Typically, expression vectors used in any of the host cells will contain sequences for plasmid maintenance and for cloning and expression of exogenous

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nucleotide sequences. such sequences, collectively referred to as "flanking sequences" in certain embodiments will typically include one or more of the following nucleotides: a promoter, one or more
5 enhancer sequences, an origin of replication, a transcriptional termination sequence, a complete intron sequence containing a donor and acceptor splice site, a leader sequence for secretion, a ribosome binding site, a polyadenylation sequence, a polylinker region for
10 inserting the nucleic acid encoding the polypeptide to be expressed, and a selectable marker element.

Flanking sequences may be homologous (i.e., from the same species and/or strain as the host cell), heterologous (i.e., from a species other than the host
15 cell species or strain), hybrid (i.e., a combination of flanking sequences from more than one source), or synthetic, or native sequences which normally function to regulate OPG and/or Fc protein expression. As such, the source of flanking sequences may be any prokaryotic
20 or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the flanking sequences is functional in, and can be activated by, the host cell machinery.

A leader, or signal, sequence may be used to
25 direct an OPG fusion polypeptide out of the host cell. The signal sequence is most commonly positioned directly at the 5' end of an OPG fusion polypeptide coding region. Many signal sequences have been identified, and any of them that are functional in the
30 selected host cell may be used in conjunction with nucleic acid sequences encoding OPG fusion proteins. For example, a signal sequence may be homologous (naturally occurring) or heterologous to the OPG or Fc gene or cDNA. Additionally, a signal sequence may be
35 chemically synthesized using methods set forth above. In most cases, secretion of an OPG polypeptide from the

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host cell via the presence of a signal peptide will result in the removal of the signal peptide from the fusion polypeptide.

The signal sequence may be a component of the vector, or it may be a part of OPG DNA that is inserted into the vector. For example, OPG DNA encodes a signal sequence at the amino terminus of the protein that is cleaved during post-translational processing of the molecule to form the mature protein (see Figure 2).
5 Included within the scope of this invention are OPG nucleotides with the native signal sequence as well as OPG nucleotides wherein the native signal sequence is deleted and replaced with a heterologous signal sequence. A heterologous signal sequence selected
10 should be one that is recognized and processed, i.e., cleaved by a signal peptidase, by the host cell. In one embodiment, a heterologous signal sequence is the OPG signal sequence as described in WO97/23614. For prokaryotic host cells that do not recognize and
15 process the native OPG signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, or heat-stable enterotoxin II leaders. For yeast secretion, the
20 native OPG signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the native signal sequence is satisfactory, although other mammalian signal sequences may be suitable.

30 Preferred vectors for practicing this invention are those which are compatible with bacterial, insect, and mammalian host cells. Such vectors include, *inter alia*, pCRII, pCR3, and pCDNA3.1 (Invitrogen Company, San Diego, CA), pBSII (Stratagene Company, La Jolla, CA), pET15b (Novagen, Madison, WI),
35 pGEX (Pharmacia Biotech, Piscataway, NJ), pEGFP-N2

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(Clontech, Palo Alto, CA), pETL (BlueBacII; Invitrogen), pDSR α 2 (PCT Publication No. WO90/14363) and pFastBacDual (Gibco/BRL, Grand Island, NY).

Additional possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the selected host cell. Such vectors include, but are not limited to plasmids such as Bluescript[®] plasmid derivatives (a high copy number ColE1-based phagemid, Stratagene Cloning Systems Inc., La Jolla CA), PCR cloning plasmids designed for cloning Taq-amplified PCR products (e.g., TOPO[™] TA Cloning[®] Kit, PCR2.1[®] plasmid derivatives, Invitrogen, Carlsbad, CA), and mammalian, yeast or virus vectors such as a baculovirus expression system (pBacPAK plasmid derivatives, Clontech, Palo Alto, CA). The recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, or other known techniques. After the vector has been constructed and a nucleic acid molecule encoding an OPG fusion polypeptide has been inserted into the proper site of the vector, the completed vector may be inserted into a suitable host cell for amplification and/or polypeptide expression.

Host cells may be prokaryotic host cells (such as *E. coli*) or eukaryotic host cells (such as a yeast cell, an insect cell, or a vertebrate cell). The host cell, when cultured under appropriate conditions, synthesizes an OPG polypeptide which can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly from the host cell producing it (if it is not secreted). Selection of an appropriate host cell will depend upon various factors, such as desired expression levels, polypeptide modifications that are desirable or necessary for

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activity, such as glycosylation or phosphorylation, and ease of folding into a biologically active molecule.

Suitable host cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO) (ATCC #CCL61 and Urlaub et al., Proc. Natl. Acad. Sci. USA 77, 4216-4220 (1980)), human embryonic kidney (HEK) 293 or 293T cells (ATCC #CRL1573), or 3T3 cells (ATCC #CRL1658). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. Other suitable mammalian cell lines, are the monkey COS-1 and COS-7 cell lines (ATCC #CRL1651), and the CV-1 cell line (ATCC #CCL70). Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene, or may contain a dominantly acting selection gene. Other suitable mammalian cell lines include but are not limited to, mouse neuroblastoma N2A cells, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines. Each of these cell lines is known by and available to those skilled in the art.

Similarly useful as host cells suitable for the present invention are bacterial cells. For example, the various strains of *E. coli* (e.g., HB101, DH5a, DH10, and MC1061) are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Pseudomonas spp.*, other *Bacillus spp.*, *Streptomyces spp.*, and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for

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expression of the polypeptides of the present invention. Preferred yeast cells include, for example, *Saccharomyces cerevisiae*.

- Additionally, where desired, insect cell systems may be utilized in the methods of the present invention. Such systems are described for example in Kitts et al. (Biotechniques, 14, 810-817 (1993)), Lucklow (Curr. Opin. Biotechnol., 4, 564-572 (1993)) and Lucklow et al. (J. Virol., 67, 4566-4579 (1993)).
- 10 Preferred insect cells are Sf-9 and Hi5 (Invitrogen, Carlsbad, CA).

- Transformation or transfection of an expression vector for an OPG polypeptide into a selected host cell may be accomplished by well known methods including methods such as calcium chloride, electroporation, microinjection, lipofection or the DEAE-dextran method. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan, and are set forth, for example, in Sambrook et al., supra.
- 15
20

Polypeptide Production

- Host cells comprising by transformation or transfection an expression vector encoding an OPG fusion polypeptide may be cultured using standard media known to the skilled artisan. The media will usually contain all nutrients necessary for the growth and survival of the cells. Suitable media for culturing *E. coli* cells are for example, Luria Broth (LB) and/or Terrific Broth (TB). Suitable media for culturing eukaryotic cells are RPMI 1640, MEM, DMEM, all of which may be supplemented with serum and/or growth factors as required by the particular cell line being cultured. A suitable medium for insect cultures is Grace's medium supplemented with yeastolate, lactalbumin hydrolysate,
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and/or fetal calf serum as necessary (Gibco Life Technologies, Gaithersburg, MD).

Typically, an antibiotic or other compound useful for selective growth of transfected or transformed cells is added as a supplement to the media. The compound to be used will be dictated by the selectable marker element present on the plasmid with which the host cell was transformed. For example, where the selectable marker element is kanamycin resistance, the compound added to the culture medium will be kanamycin; where the selectable marker element is ampicillin resistance, the compound added to the culture medium will be ampicillin.

The amount of an OPG fusion polypeptide produced by a host cell can be evaluated using standard methods known in the art. Such methods include, without limitation, Western blot analysis, SDS-polyacrylamide gel electrophoresis, non-denaturing gel electrophoresis, HPLC separation, immunoprecipitation, and/or activity assays such as DNA binding gel shift assays.

Where an OPG fusion polypeptide is prepared without a tag attached, and no antibodies are available, other well known procedures for purification can be used. Such procedures include, without limitation, ion exchange chromatography, molecular sieve chromatography, HPLC, native gel electrophoresis in combination with gel elution, and preparative isoelectric focusing ("Isoprime" machine/technique, Hoefer Scientific). In some cases, two or more of these techniques may be combined to achieve increased purity.

If an OPG fusion polypeptide is produced intracellularly, the intracellular material (including inclusion bodies for gram-negative bacteria) can be extracted from the host cell using any standard

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technique known to the skilled artisan. For example, the host cells can be lysed to release the contents of the periplasm/cytoplasm by French press, homogenization, and/or sonication followed by centrifugation.

If an OPG fusion polypeptide has formed inclusion bodies in the cytosol, the inclusion bodies can often bind to the inner and/or outer cellular membranes and thus will be found primarily in the pellet material after centrifugation. The pellet material can then be treated at pH extremes or with chaotropic agent such as a detergent, guanidine, guanidine derivatives, urea, or urea derivatives in the presence of a reducing agent such as dithiothreitol at alkaline pH or tris carboxyethyl phosphine at acid pH to release, break apart, and solubilize the inclusion bodies. An OPG fusion polypeptide in its now soluble form can then be analyzed using gel electrophoresis, immunoprecipitation or the like. If it is desired to isolate an OPG polypeptide, isolation may be accomplished using standard methods such as those set forth below and in Marston et al. (Meth. Enz., 182, 264-275 (1990)).

In some cases, an OPG fusion polypeptide may not be biologically active upon isolation. Various methods for "refolding" or converting the polypeptide to its tertiary structure and generating disulfide linkages, can be used to restore biological activity. Such methods include exposing the solubilized polypeptide to a pH usually above 7 and in the presence of a particular concentration of a chaotrope. The selection of chaotrope is very similar to the choices used for inclusion body solubilization, but usually the chaotrope is used at a lower concentration and is not necessarily the same as chaotropes used for the solubilization. In most cases the refolding/oxidation

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solution will also contain a reducing agent or the reducing agent plus its oxidized form in a specific ratio to generate a particular redox potential allowing for disulfide shuffling to occur in the formation of the protein's cysteine bridge(s). Some of the commonly used redox couples include cysteine/cystamine, glutathione (GSH)/dithiobis GSH, cupric chloride, dithiothreitol (DTT)/dithiane DTT, and 2-mercaptoethanol (β ME)/dithio- β (ME). In many instances, a cosolvent may be used or may be needed to increase the efficiency of the refolding and the more common reagents used for this purpose include glycerol, polyethylene glycol of various molecular weights, arginine and the like.

15

Derivatives

The present OPG fusion proteins, and variants and fragments thereof, are derivatized by the attachment of one or more chemical moieties. As an example, a fusion of OPG and Fc polypeptide may be derivatized on either OPG or Fc moieties, or both. These chemically modified derivatives may be further formulated for intraarterial, intraperitoneal, intramuscular subcutaneous, intravenous, oral, nasal, pulmonary, topical or other routes of administration as discussed below. Chemical modification of biologically active proteins has been found to provide additional advantages under certain circumstances, such as increasing the stability and circulation time of the therapeutic protein and decreasing immunogenicity. See, U.S. Patent No. 4,179,337. For a review, see Abuchowski et al., in *Enzymes as Drugs*. (J. S. Holcberg and J. Roberts, eds. pp. 367-383 (1981)); Francis et al., *supra*.

35

The chemical moieties suitable for such derivatization may be selected from among various water

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soluble polymers. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. For the present proteins, the effectiveness of the derivatization may be ascertained by administering the derivative, in the desired form (i.e., by osmotic pump, or, more preferably, by injection or infusion, or, further formulated for oral, pulmonary or nasal delivery, for example), and observing biological effects as described herein.

The water soluble polymer may be selected from the group consisting of, for example, polyethylene glycol, copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohol. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. Also, succinate and styrene may also be used. In addition, polyaminoacids may be selected from the group consisting of serum album (such as human serum albumin), or other polyaminoacids, e.g. lysines.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 2 kDa and about 100 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated

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molecular weight) for ease in handling and manufacturing.

The number of polymer molecules so attached to an OPG fusion polypeptide may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or some combination of derivatization, with the same or different chemical moieties (e.g., polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to protein (or peptide) molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by factors such as the desired degree of derivatization (e.g., mono, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

The chemical moieties should be attached to an OPG fusion protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art (EP 0401384 herein incorporated by reference (coupling PEG to G-CSF); Malik et al., Exp. Hematol. 20, 1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride)). For example, polyethylene glycol may be covalently bound through amino acid residues having a free amino group (e.g., lysine, arginine, or the N-terminal amino acid residue) or a free carboxyl group (e.g., aspartic acid, glutamic acid, and the C-terminal amino acid residue). Amino acid residues having a free sulfhydryl group (e.g., cysteine) may also be used. Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group.

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Attachment at residues important for receptor binding should be avoided if receptor binding is desired.

One may specifically desire N-terminally chemically modified OPG fusion protein. Using polyethylene glycol as an example of the chemical moiety, a preparation of substantially N-terminally pegylated OPG fusion polypeptide may be obtained by derivatizing the polypeptide at free amino groups and separating N-terminally pegylated material from a population of pegylated protein molecules. Alternatively, selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

An N-terminally monopegylated derivative is preferred for ease in production of a therapeutic. N-terminal pegylation ensures a homogenous product as characterization of the product is simplified relative to di-, tri- or other multi-pegylated products. The use of the above reductive alkylation process for preparation of an N-terminal product is preferred for ease in commercial manufacturing.

30

Uses of the polypeptides

The fusion polypeptides of the invention are used in the prevention and/or treatment of loss of bone mass; prevention and/or treatment of replacement of structurally sound bone with disorganized bone; or in the prevention of metastasis to bone. Bone loss is

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manifested in a variety of conditions including the following: Osteoporosis, such as primary osteoporosis, endocrine osteoporosis (hyperthyroidism, hyperparathyroidism, Cushing's syndrome, and acromegaly), hereditary and congenital forms of osteoporosis (osteogenesis imperfecta, homocystinuria, Menkes' syndrome, and Riley-Day syndrome) and osteoporosis due to immobilization of extremities; Paget's disease of bone (osteitis deformans) in adults and juveniles; Osteomyelitis, or an infectious lesion in bone, leading to bone loss; Hypercalcemia resulting from solid tumors (breast, lung and kidney) and hematologic malignancies (multiple myeloma, lymphoma and leukemia), idiopathic hypercalcemia, and hypercalcemia associated with hyperthyroidism and renal function disorders; Osteopenia following surgery, induced by steroid administration, and associated with disorders of the small and large intestine and with chronic hepatic and renal diseases; Osteonecrosis, or bone cell death, associated with traumatic injury or nontraumatic necrosis associated with Gaucher's disease, sickle cell anemia, systemic lupus erythematosus and other conditions; Bone loss due to rheumatoid arthritis; Periodontal bone loss; Osteoarthritis; Prosthetic loosening; and Osteolytic metastasis. Replacement of structurally sound bone with disorganized structurally incompetent bone is seen in Paget's disease of bone (osteitis deformans) in adults and juveniles; hyperparathyroidism, in congenital bone disorders such as fibrous dysplasia, and in osteosclerotic bone metastases:

In an embodiment of the invention, the OPG fusion polypeptides of the invention, by virtue of increased activity and circulating half-life, are advantageously used to treat bone loss, and especially

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bone loss resulting from osteolytic destruction of bone caused by malignant or metastatic tumors. OPG polypeptides of the invention may be used to treat bone loss associated with breast, prostate, thyroid, kidney, lung, esophageal, rectal, bladder, cervical, ovarian and liver cancers as well as cancer of the gastrointestinal tract. Also included is bone loss associated with certain hematological malignancies such as multiple myeloma and lymphomas such as Hodgkin's Disease.

The OPG fusion proteins of the invention are administered alone or in combination with other therapeutic agents, in particular, in combination with other cancer therapy agents. Such agents generally include radiation therapy or chemotherapy. Chemotherapy may involve treatment with one or more of the following: anthracyclines, taxol, tamoxifene, doxorubicin, 5-fluorouracil, and other drugs known to the skilled worker. In one embodiment, the cancer therapy agent is a luteinizing hormone-releasing hormone (LHRH) antagonist, preferably a peptide antagonist. More preferably, an LHRH antagonist is a decapeptide comprising the following structure:

A-B-C-D-E-F-G-H-I-J

wherein

A is pyro-glu, Ac-D-Nal, Ac-D-Qal; Ac-Sar, or Ac-D-Pal;

B is His or 4-Cl-D-Phe;

C is Trp, D-Pal, D-Nal, L-Nal-D-Pal(N-O), or D-Trp;

D is Ser;

E is N-Me-Ala, Tyr, N-Me-Tyr, Ser, Lys(iPr), 4-Cl-Phe, His, Asn, Met, Ala, Arg or Ile;

F is

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wherein R and X are independently, H and alkyl;
and Y comprises a small polar entity.

5 G is Leu or Trp;

H is Lys(iPr), Gln, Met, or Arg;

I is Pro; and

J is Gly-NH₂ or D-Ala-NH₂;

or a pharmaceutically acceptable salt thereof.

10 In another embodiment, an LHRH antagonist
comprises the peptide:

N-Ac-D-Nal-4-Cl-Phe-D-Pal-Ser-N-Me-Tyr-D-Asn-Leu-
Lys(iPr)-Pro-D-Ala-NH₂.

Standard abbreviations and conventions are
15 used herein and the following non-standard residues and
moieties are abbreviated as follows:

	Nal	3-(2-naphthyl)alaninyl
	4-Cl-Phe	(4'-chlorophenyl)alaninyl
20	Pal	3-(3'-pyridyl)alaninyl
	Pal(N-O)	3-(3'-pyridine-N-oxide)alaninyl
	iPr-Lys	N-epsilon-2-propyl-lysiny
	Qal	3-(2'-quinolinyl)alaninyl

Alternative forms of LHRH antagonist
25 decapeptides are also encompassed by the invention.
Such decapeptides are described in U.S. Patent No.
5,843,901 hereby incorporated by reference.

Also included are therapeutic antibodies
including mouse, mouse-human chimeric, CDR-grafted,
30 humanized or fully human antibodies, or synthetic
antibodies such as those selected by screening antibody
libraries. Examples of such antibodies include those
which bind to cell surface proteins Her2, CDC20, CDC33,

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mucin-like glycoprotein and epidermal growth factor receptor (EGFR) present on tumor cells and optionally induce a cytostatic and/or cytotoxic effect on tumor cells displaying these proteins. Examples of such

5 antibodies include HERCEPTIN for treatment of breast cancer and RITUXAN for the treatment of non-Hodgkin's lymphoma. Also included as cancer therapy agents are polypeptides which selectively induce apoptosis in tumor cells, such as the TNF-related polypeptide TRAIL.

10 OPG fusion proteins may be administered prior to, concurrent with, or subsequent to treatment with a cancer therapy agent. OPG fusion proteins may be administered prophylactically to prevent or mitigate the onset of bone loss by metastatic cancer or may be given

15 for the treatment of an existing condition of bone loss due to metastasis.

OPG fusion polypeptides of the invention may be used to prevent and/or treat bone loss

20 associated with multiple myeloma or to prevent and/or treat the disease itself. Multiple myeloma is a B cell derived tumor that results in significant morbidity and mortality. The most striking common clinical manifestation is the focal bone loss due to increased

25 osteoclast activation in localized regions. The majority of myeloma patients (~95%) usually present with destructive bone lesions visible by radiological analysis, and suffer from extreme, intractable skeletal pain. Patients with myeloma are particularly

30 susceptible to pathological fractures of involved bone, which occur either spontaneously or due to trivial injury. The skeletal lesions that occur during myeloma not only lead to bone fractures, but also deformity and occasionally nerve compression, particularly in the

35 vertebral spine. In some patients, a pathological increase in serum calcium (hypercalcemia) occurs, and

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can cause significant problems during disease treatment. OPG may be administered to patients to block bone resorption and release of calcium, thereby reducing the risk of fractures and spinal deformity.

5 Myeloma cells do not directly participate in bone destruction, but instead produce extracellular signals that lead to osteoclast differentiation and activation. Osteoclasts in turn produce the highest levels of the potent cytokine IL-6 of any cell type in
10 the body, particularly when they become activated. IL-6 is a B-cell growth factor, and is required for the growth of both murine and human myeloma cells in vitro. A TNF-related protein OPG ligand (OPGL) is responsible for inducing osteoclast differentiation and activation
15 (See WO98/46751). Myeloma cells may either directly or indirectly produce this factor to osteoclasts, resulting in local bone lysis surrounding the myeloma cells embedded in bone marrow spaces. The normal osteoclasts adjacent to the myeloma cell in turn
20 produce IL-6, leading to local expansion of the tumor cells. Myeloma cells expand in a clonal fashion and occupy bone spaces that are being created by inappropriate bone resorption.

 It has been observed that OPG administration
25 in rodents induces rapid death of the osteoclast population. A reduction in osteoclasts could eliminate the increase in IL-6 production by osteoclasts and affect the growth and survival of myeloma cells within trabecular bone. Thus, OPG treatment in myeloma
30 patients would not only block the hyper resorption of bone, but could also affect the expansion and survival of the tumor itself. B-cells are known to express the receptor for OPGL, referred to as osteoclast differentiation and activation receptor, or ODAR.
35 Myeloma cells also express ODAR, and in addition may produce OPGL. The expression of both OPGL and ODAR on

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the same cell population may create an autocrine stimulus that affects survival of the myeloma cell. Thus, OPG treatment could directly affect tumor cell survival, thus decreasing or eliminating, the tumor burden seen in myeloma patients.

Pharmaceutical Compositions

The present invention also provides for pharmaceutical compositions comprising OPG fusion proteins, and variants, fragments and derivatives thereof. Such pharmaceutical compositions may be for administration for injection, or for oral, pulmonary, nasal, transdermal or other forms of administration. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of an OPG fusion protein of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. An effective or a therapeutically effective amount of an OPG fusion protein is an amount sufficient to reduce the rate and/or extent of bone loss as determined by assays and procedures described herein.

Pharmaceutical compositions of the invention include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimersol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation. Such

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compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives.

- See, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form. Implantable sustained release formulations are also contemplated, as are transdermal formulations.

- Contemplated for use herein are oral solid dosage forms, which are described generally in Remington's Pharmaceutical Sciences, 18th Ed. 1990 (Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (e.g., U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, K. In: *Modern Pharmaceutics* Edited by G. S. Banker and C. T. Rhodes Chapter 10, 1979, herein incorporated by reference. In general, the formulation will include an OPG fusion protein, or a variant, fragment or derivative thereof, and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

- An OPG fusion protein may optionally be chemically modified so that oral delivery of the derivative is efficacious. Generally, the chemical modification contemplated is the attachment of at least

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- one moiety to the protein (or peptide) molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase
- 5 in overall stability of the protein and increase in circulation time in the body. Examples of such moieties include polyethylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl
- 10 pyrrolidone and polyproline. Abuchowski and Davis, Soluble Polymer-Enzyme Adducts. In: "Enzymes as Drugs", Hochenberg and Roberts, eds., Wiley-Interscience, New York, NY, (1981), pp 367-383; Newmark, et al., J. Appl. Biochem. 4: 185-189 (1982).
- 15 Other polymers that could be used are poly-1,3-dioxolane and poly-1,3,6-tioxocane. Preferred for pharmaceutical usage, as indicated above, are polyethylene glycol moieties.

- To ensure resistance to degradation in the
- 20 stomach following oral administration, a coating impermeable to at least pH 5.0 is essential. Examples of the more common inert ingredients that are used as enteric coatings for oral formulations are cellulose acetate trimellitate (CAT), hydroxypropylmethyl
- 25 cellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films.

- 30 An OPG fusion protein may be included in a formulation as fine multiparticulates in the form of granules or pellets of particle size about 1 mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or
- 35 even as tablets.

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- Pharmaceutical compositions of the invention include diluents such as carbohydrates, especially mannitol, α -lactose, anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain
- 5 inorganic salts may be also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.
- 10 Disintegrants may be included in solid dosage formulations. Materials used as disintegrates include but are not limited to starch including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose,
- 15 ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and
- 20 as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.
- Binders may be used to form hard tablets and include materials from natural products such as acacia,
- 25 tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.
- 30 Lubricants that may be added to a formulation include but are not limited to; stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also
- 35 be used such as sodium lauryl sulfate, magnesium lauryl

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sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of an OPG fusion protein composition, a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethonium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative either alone or as a mixture in different ratios.

Additives which potentially enhance uptake of a protein are, for instance, the fatty acids oleic acid, linoleic acid and linolenic acid.

A controlled release formulation may be desirable. An OPG fusion protein may be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms e.g., gums. Slowly degenerating matrices may also be incorporated into the formulation, e.g., alginates, polysaccharides. Another form of a controlled release of this therapeutic is by a method based on the Oros therapeutic system (Alza Corp.), i.e., the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out

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through a single small opening due to osmotic effects. Some enteric coatings also have a delayed release effect.

Other coatings may be used in a formulation.

- 5 For example, a film coated tablet may comprise materials from two different groups. The first group includes nonenteric materials such as methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl
- 10 methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid. A mix of materials might be used to provide the optimum film coating.
- 15 Film coating may be carried out in a pan coater or in a fluidized bed or by compression coating.

- Also contemplated herein is pulmonary delivery of an OPG polypeptide or fusion protein. The protein is delivered to the lungs of a mammal while
- 20 inhaling and traverses across the lung epithelial lining to the blood stream. (Other reports of this include Adjei *et al.*, *Pharmaceutical Research* 7: 565-569 (1990); Adjei *et al.*, *International Journal of Pharmaceutics* 63: 135-144 (1990) (leuprolide
- 25 acetate); Braquet *et al.*, *Journal of Cardiovascular Pharmacology* 13 (suppl. 5): s.143-146 (1989) (endothelin-1); Hubbard *et al.*, *Annals of Internal Medicine* 3: 206-212 (1989) (α1-antitrypsin); Smith *et al.*, *J. Clin. Invest.* 84: 1145-1146
- 30 (1989) (α1-proteinase); Oswein *et al.*, "Aerosolization of Proteins", *Proceedings of Symposium on Respiratory Drug Delivery II*, Keystone, Colorado, March, 1990 (recombinant human growth hormone); Debs *et al.*, *The Journal of Immunology* 140: 3482-3488 (1988) (interferon

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α and tumor necrosis factor α) and U.S. Patent No. 5,284,656 (granulocyte colony stimulating factor).

Contemplated for use in the practice of this invention are a wide range of mechanical devices
5 designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art. Some specific examples of commercially available devices
10 suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri; the Acorn II nebulizer, manufactured by Marquest Medical Products, Englewood, Colorado; the Ventolin metered dose inhaler,
15 manufactured by Glaxo Inc., Research Triangle Park, North Carolina; and the Spinhaler powder inhaler, manufactured by Fisons Corp., Bedford, Massachusetts.

All such devices require the use of formulations suitable for the dispensing of an OPG
20 fusion protein, or a variant, fragment or derivative thereof. Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to diluents, adjuvants and/or carriers useful in therapy.

25 An OPG fusion protein should most advantageously be prepared in particulate form with an average particle size of less than 10 μm (or microns), most preferably 0.5 to 5 μm , for most effective delivery to the distal lung.

30 Carriers include carbohydrates such as trehalose, mannitol, xylitol, sucrose, lactose, and sorbitol. Other ingredients for use in formulations may include DPFC, DOPE, DSPC and DOPC. Natural or synthetic surfactants may be used. Polyethylene glycol
35 may be used (even apart from its use in derivatizing

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the protein or analog). Dextran, such as cyclodextran, may be used. Bile salts and other related enhancers may be used. Cellulose and cellulose derivatives may be used. Amino acids may be used, such as use in a buffer formulation. The use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is also contemplated.

Nasal delivery of an OPG fusion protein is also contemplated. Nasal delivery allows the passage of the protein to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran. Delivery via transport across other mucus membranes is also contemplated.

Dosages

OPG fusion polypeptides of the invention are administered in a therapeutically effective amount to prevent and/or treat loss of bone associated with metastatic bone disease. A "therapeutically effective amount" of an OPG fusion polypeptide is that amount which reduces the loss of bone mass. Bone mass is measured by a variety of known methods such as single photon absorptiometry (SPA), dual photon absorptiometry (DPA), dual energy X-ray absorptiometry (DEXA), quantitative computed tomography (QCT), and ultrasonography (See Johnston et al. in Primer on the Metabolic Bone Disease and Disorders of Mineral Metabolism, 2nd ed., M.J. Favrus, ed. Raven Press pp. 237-146)). One skilled in the art can use these methods to determine a therapeutically effective amount of an OPG fusion polypeptide. A therapeutically effective amount may also be determined by measuring

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- changes in biochemical markers for bone turnover, such as serum osteocalcin, serum alkaline phosphatase, serum procollagen I extension peptides, urinary or serum C-terminal or N-terminal telopeptide of collagen, urinary
- 5 calcium, hydroxyproline and urinary pyridinoline and deoxypyridinoline. It is generally recognized that a decrease in the levels of the aforementioned biochemical markers indicates that bone resorption is decreased and bone loss is being reduced.
- 10 Alternatively, a therapeutically effective amount of an OPG fusion polypeptide may also be determined by measuring an increase in bone strength, in particular an increase in torsional (twisting) strength of bone.

- In general, a therapeutically effective
- 15 amount of an OPG fusion polypeptide is from about 0.1 mg/kg to about 10 mg/kg, preferably from about 1mg/kg to about 10 mg/kg. By virtue of the increased half-life of an OPG fusion polypeptide, and especially a fusion of OPG to an immunoglobulin Fc region,
- 20 administration will be less frequent than for an unmodified OPG polypeptide. The frequency of administration may be about one time per month, or alternatively, one time every two months, or one time every three months. It will be appreciated by one
- 25 skilled in the art that the exact dosage and frequency of administration will depend upon several factors, including formulation, route of administration, condition being treated, and so forth, and may be readily determined by the skilled worker.

- 30 The amount of OPG fusion protein which has been administered may be determined using diagnostic assays for the fusion protein. Such diagnostic assays may be in the form of an antibody assay, such as an antibody sandwich assay, wherein the antibody
- 35 specifically binds to the OPG fusion protein, but does not bind to endogenous, naturally circulating OPG or to

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a form of the heterologous protein fused to OPG which may also circulate naturally, such as an Fc region of a naturally occurring antibody. Antibody based assays for determining OPG fusion protein levels may be
5 carried out in a variety of formats that are known to one skilled in the art.

The following examples are offered to more fully illustrate the invention, but are not construed
10 as limiting the scope thereof.

EXAMPLE 1

Construction and Expression of
OPG Fusion Polypeptides

Plasmids encoding OPG[1-194]-Fc, OPG[1-201]-Fc, OPG[1-194]-FcΔC, OPG[1-201]-FcΔC, OPG[1-194]-FcG₄, and metFcΔC-OPG[22-194] for use in producing the
20 corresponding OPG fusion polypeptides are constructed generally as described in W097/23614 and in copending U.S. Serial No. _____, filed September __, 1999, both of which are incorporated by reference. The polypeptide sequences are shown in Figures 3-8,
25 respectively.

Expression of an OPG fusion polypeptide in mammalian and bacterial host cells was carried out generally as described in W097/23614.

EXAMPLE 2

OPG Activity in a Breast Cancer Model
for Lytic Bone Disease

Female Balb/c nu/nu mice aged 7-8 weeks were
35 injected with human MDA-MB-231 breast cancer cells (1.0

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x 10^5 cells/mouse; ATCC accession no. HTB-26) directly into the systemic circulation via the left ventricle. Immediately following tumor inoculation, the mice were treated by intravenous injection with either phosphate buffered saline (PBS) or met FcAC-OPG[22-194] (25mg/kg) three times per week for 4 weeks. The number of lesions/mouse was assessed from radiographs. Bone, heart, lung, liver, kidneys, adrenals, ovaries, brain, pancreas and spleen were evaluated histologically for the presence of tumor foci as described below.

As shown in Figure 9, radiographic lesions are apparent in the long bones 4 weeks after inoculation with MDA-MB-231 cells. The associated bone destruction is completely inhibited by intravenous administration of met FcAC-OPG[22-194] at a dose of 25mg/kg three times per week commencing at the time of inoculation (6.2 ± 0.8 vs. 0.0 ± 0.0 lesions/mouse, $p < 0.001$).

20

EXAMPLE 3

OPG Activity in a Mouse Adenocarcinoma Model
for Lytic Bone Disease

Female CDF1 mice aged 7-8 weeks were injected with murine C26-DCT adenocarcinoma cells (obtained from the Tumor Repository of the National Cancer Institute; 1.0×10^5 cells/mouse) directly into the systemic circulation via the left ventricle. Immediately following tumor inoculation, mice were treated by intravenous injection with either PBS or met FcAC-OPG[22-194] (25mg/kg) every 3 days for 9 days. On day 10 the mice were radiographed and tissues were sampled for histological evaluation.

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As shown in Figure 9, radiographic lesions are evident 10 days after intra-cardiac inoculation of C26-DCT cells (3.1 ± 0.6 lesions/mouse) and bone destruction is inhibited by intravenous injection of
5 FcdC-OPG[22-194].

In an additional study, mice were inoculated with C26-DCT cells, as above, and treated by intravenous injection with either PBS or met Fc-OPG[22-194] at 3, 1, 0.3, or 0.1mg/kg every 3 days for 9 days.
10 On day 10 the mice were radiographed. Radiographs were assessed and tissues were handled as described below. Radiographic lesions were reduced in a dose dependent manner by intravenous met FcAC-OPG [22-194] treatment. (Figure 10)

15

EXAMPLE 4

OPG Activity in a Mouse Adenocarcinoma Model
for Hypercalcemia

20

Male Balb/c x DBA/2 (CDF1) mice at 10-12 weeks of age were purchased from either Harlan Sprague Dawley (San Diego, CA) or Charles River (Wilmington, MA).

25

The C-26 tumor, originally induced in a female Balb/c mouse by repeated intrarectal instillation of *N*-nitroso-*N*-methylurethan (NMU) (Corbett et al. Cancer Res. 35, 2434-2439 (1975)), was obtained from the Tumor Repository of the National
30 Cancer Institute in the form of tissue fragments. The fragments were mechanically disrupted and placed into culture under standard tissue culture conditions (37°C, 5-6% CO₂), which gave rise to an adherent cell line that could be continually propagated and was
35 subsequently frozen down as such. The media employed

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for passage of the cells in vitro is DMEM plus 10% FCS, 1x pen-strep/glutamine and 1x nonessential amino acids. A separate frozen vial of cells from a common stock was used for the initiation of all experiments. After
5 initial reestablishment in culture, cells were harvested by trypsinization, followed by several washes and resuspension in DMEM with no additives to a concentration of 2.5×10^6 cells/ml. Animals were injected subcutaneously (SQ) over a shaved area of the
10 right flank with 0.2 cc (0.5×10^6 cells). Under these conditions tumor development was found to be very consistent with minimal variability.

Treatment began on either day 8 (prevention studies) or when blood ionized calcium levels reached a
15 level >1.60 mmol/L (treatment studies). Treatment lasted for 8 days in the prevention studies and for 4 days in the treatment studies. In the prevention studies metFcΔC-OPG[22-194] was administered daily in a PBS vehicle as a subcutaneous injection in the flank.
20 In the treatment studies, met FcΔC-OPG[22-194] was administered as a single intravenous injection in PBS vehicle. In both studies normal and tumor bearing control animals received a similar injection of PBS.

During the course of the study, mice were
25 weighed daily (while on treatment), and blood ionized calcium levels were monitored either every other day in the prevention studies (prior to the drug administration on that day) or daily in the treatment studies. Blood was sampled retro-orbitally and blood
30 ionized calcium determinations were performed using a blood ionized calcium/pH analyzer (Chiron Diagnostics #634, Norwood, MA).

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Histological Evaluation

- One leg from each animal (femur and tibia connected) was harvested at the conclusion of the study. The bones were fixed in phosphate buffered zinc formalin, decalcified in formic acid and embedded in paraffin. Sections were taken through the midregion of the distal femur and proximal tibia, reacted to demonstrate tartrate resistant acid phosphatase activity (TRAP), and counterstained with hematoxylin.
- 10 In this staining procedure osteoclasts are stained red, while other cell types, bone and cartilage are stained various shades of blue.

- Measurements were made of the area just distal to the proximal tibial growth plate (in the area of the primary spongiosa), and in an area of the cortical shaft of the tibia 2mm distal to the first measurement area using an Osteomeasure bone analysis program (Osteometrics Inc., Decatur, GA). Two separate regions of the tibia were chosen for measurement so an accurate determination of the tumor induced increase in bone resorption could be obtained. The field of measurement consisted of a 1mm x 1mm square area in both locations and did not include the growth plate in the first region. The parameters measured were the number and active surface of osteoclasts, and bone surface. Results were recorded as osteoclast number (OcN), osteoclast perimeter (active osteoclast surface) (OcPm), bone perimeter (bone surface) (BPm). In order for an osteoclast to be considered for measurement, it had to be TRAP positive and in contact with a bone surface. The active osteoclast surface was the part of the osteoclast that was in direct contact with a bone surface. All measurements were made by tracing the section image onto a digitizing platen with the aid of a camera lucida attachment on the microscope.

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Results

OPG treatment moderated the effects of the C-26 adenocarcinoma on body weight loss. Mice receiving a daily 2.5mg/kg dose of OPG lost an average of 5.2 grams body weight while untreated tumor bearing animals lost an average of 6.2 grams. PBS tumor bearing mice had tumors that were $3.47 \pm 0.72\%$ vs OPG 2.5 mg/kg $3.42 \pm 0.82\%$. Weights are: PBS $0.75 \pm 0.14g$ and OPG 2.5: $0.79 \pm 0.16g$.

OPG prevents and reverses C-26 tumor induced increases in blood ionized calcium levels

When treatment was commenced on day 9 following implantation of the tumor, OPG dose-dependently inhibited the increase in whole blood ionized calcium levels induced by the tumor (see Figure 11A). Prior to commencing OPG treatment, the tumor bearing mice had slightly increased whole blood ionized calcium levels (1.34 ± 0.06 mmol/L vs 1.25 ± 0.02 mmol/L). In the vehicle treated groups these levels continued to increase throughout the experiment with maximum levels of 1.84 ± 0.12 mmol/L and reached on days 13 and 15, and decreasing slightly by day 16. The OPG treated groups demonstrated a dose dependent inhibition of this increase in whole blood ionized calcium levels throughout the treatment period, with ionized calcium levels reaching a maximum of 1.38 ± 0.06 mmol/L on day15 in the 2.5 mg/kg group. This level of calcium was moderately but significantly higher than that found in non-tumor bearing control animals. OPG treatment had no effect on calcium levels in non-tumor bearing mice.

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When mice were allowed to become frankly hypercalcemic prior to treatment, a single 5.0 mg/kg dose of OPG produced a rapid decrease in calcium levels with a significant decrease evident by 12 hours and normocalcemia achieved within 24 hours of treatment. (see Figure 11B). OPG maintained calcium levels in the normal range for the remainder of the experiment.

10 OPG prevents and reverses C-26 tumor induced increases
in osteoclast lined bone surfaces and osteoclast
numbers

Osteoclast lined surfaces and osteoclast numbers were markedly elevated in hypercalcemic mice bearing C26 tumors. Treatment with a 2.5 mg/kg dose of OPG either prior to or after the development of hypercalcemia caused almost a completed disappearance of osteoclasts. Osteoclast surface measurements, an indication of the amount of bone resorption, were significantly increased in the tumor bearing animals, 20 $8.95 \pm 2.10\%$, compared to non tumor bearing controls $3.91 \pm 1.10\%$. A 2.5 mg/kg dose of OPG given daily prior to the development of hypercalcemia dose dependently reduced these to $0.13 \pm 0.07\%$ which is significantly lower than even the non tumor bearing 25 control animals.

The number of osteoclasts per mm bone surface were also elevated in the tumor bearing animals to 4.41 ± 1.03 /mm compared to non tumor bearing controls 2.00 ± 0.52 /mm. A daily 2.5 mg/kg dose of OPG dose 30 dependently reduced the number of osteoclasts per mm to 0.12 ± 0.06 /mm which is significantly lower then even the non-tumor bearing animals.

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OPG treatment dose dependently decreases the size and number of osteoclasts

Osteoclast surface as a percent of bone surface measurements as well as the number of osteoclasts per mm bone surface were both significantly elevated in the tumor bearing control animals $8.95 \pm 1.64 \%$ and 4.12 ± 0.72 osteoclasts per mm respectively, when compared to normal non-tumor bearing animals $3.66 \pm 1.01 \%$ and 1.83 ± 0.54 osteoclasts per mm. A daily 2.5 mg/kg dose of OPG significantly reduced these values below even the normal range to $1.26 \pm 0.93\%$ and 0.73 ± 0.55 osteoclasts per mm.

15

* * *

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

20

25

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WHAT IS CLAIMED IS:

1. A method of preventing or treating lytic bone disease in a mammal comprising administering a therapeutically effective amount of an OPG polypeptide.
2. A method for preventing the metastasis of cancer to bone comprising administering a therapeutically effective amount of an OPG polypeptide.
3. A method for preventing the osteosclerotic bone metastasis comprising administering a therapeutically effective amount of an OPG polypeptide.
4. The method of Claim 1 or 2 or 3 further comprising administering a therapeutically effective amount of a cancer therapy agent.
5. The method of Claims 1 or 2 or 3 or 4 wherein the OPG polypeptide comprises an amino acid sequence as shown in Figure 2 (SEQ ID NO: 2) or a truncated polypeptide thereof.
6. The method of Claim 4 wherein the OPG polypeptide comprises a carboxy terminal truncation of part or all of amino acid residues 186-401 as shown in Figure 2 (SEQ ID NO: 2).
7. The method of Claim 4 wherein the OPG polypeptide comprises amino acid residues 22-194 inclusive as shown in Figure 2 (SEQ ID NO: 2).
8. The method of Claims 5, 6 or 7 wherein the OPG polypeptide is an OPG fusion polypeptide.

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9. The method of Claim 8 wherein the OPG fusion polypeptide comprises a fusion of an Fc region to the N-terminal or C-terminal end of the OPG polypeptide.

5

10. The method of Claim 9 wherein the OPG fusion polypeptide comprises an Fc region fused to amino acid residues 22-194 of Figure 2 (SEQ ID NO: 2).

10

11. The method of Claim 10 wherein the OPG fusion polypeptide consists of the amino acid sequence as shown in Figure 5 or in Figure 8 (SEQ ID NO: 5 or 8).

15

12. The method of Claim 1 or 2 or 3 wherein the OPG polypeptide is administered prior to, concurrent with, or subsequent to administration of a cancer therapy agent.

20

13. The method of Claim 1 or 3 wherein lytic bone disease occurs in conjunction with cancer which has metastasized to bone.

25

14. The method of Claim 13 wherein the cancer is selected from the group consisting of breast cancer, prostate cancer, thyroid cancer, cancer of the kidney, lung cancer, esophageal cancer, rectal cancer, bladder cancer, cervical cancer, ovarian cancer, liver cancer, cancer of the gastrointestinal tract, multiple myeloma, and lymphoma.

30

15. The method of Claim 1 or 2 or 3 wherein the cancer therapy agent is selected from the group consisting of radiation, chemotherapy, antibodies, or non-antibody polypeptides.

35

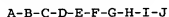
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16. The method of Claim 15 wherein chemotherapy comprises anthracyclines, taxol, tamoxifene, doxorubicin, and 5-fluorouracil.

5 17. The method of Claim 15 wherein the antibodies bind to Her2, CDC20, CDC33, mucin-like glycoprotein, or epidermal growth factor receptor (EGFR) on the surface of tumor cells.

10 18. The method of Claim 15 wherein the cancer therapy agent comprises a luteinizing hormone-releasing hormone (LHRH) antagonist.

19. The method of Claim 18 wherein the LHRH antagonist comprises the following structure:



wherein

A is pyro-glu, Ac-D-Nal, Ac-D-Qal; Ac-Sar, or Ac-D-Pal;

20 B is His or 4-Cl-D-Phe;

C is Trp, D-Pal, D-Nal, L-Nal-D-Pal(N-O), or D-Trp;

D is Ser;

25 E is N-Me-Ala, Tyr, N-Me-Tyr, Ser, Lys(iPr), 4-Cl-Phe, His, Asn, Met, Ala, Arg or Ile;

F is



wherein R and X are independently, H and alkyl; and Y comprises a small polar entity.

30 G is Leu or Trp;

H is Lys(iPr), Gln, Met, or Arg;

I is Pro; and

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J is Gly-NH₂ or D-Ala-NH₂;
or a pharmaceutically acceptable salt thereof.

20. The method of Claim 18 wherein the LHRH
5 antagonist comprises the peptide: N-Ac-D-Nal-4-Cl-Phe-
D-Pal-Ser-N-Me-Tyr-D-Asn-Leu-Lys(iPr)-Pro-D-Ala-NH₂.

21. The method of Claims 1 or 2 or 3 or 8
wherein the therapeutically effective amount of an OPG
10 polypeptide or an OPG fusion polypeptide is between
0.1mg/kg and 10mg/kg.

22. A method of preventing or treating
multiple myeloma comprising administering
15 therapeutically effective amount of an OPG polypeptide.

FIG. 1

Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala
 1 5 10 15
 Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
 20 25 30
 Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
 35 40 45
 Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
 50 55 60
 Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
 65 70 75 80
 Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
 85 90 95
 Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
 100 105 110
 Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
 115 120 125
 Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr
 130 135 140
 Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
 145 150 155 160
 Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
 165 170 175
 Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
 180 185 190
 Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
 195 200 205
 Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
 210 215 220
 Ser Leu Ser Leu Ser Pro Gly Lys
 225 230

FIG. 2A

Met	Asn	Lys	Trp	Leu	Cys	Cys	Ala	Leu	Leu	Val	Leu	Leu	Asp	Ile	Ile
1				5					10					15	
Glu	Trp	Thr	Thr	Gln	Glu	Thr	Leu	Pro	Pro	Lys	Tyr	Leu	His	Tyr	Asp
			20					25					30		
Pro	Glu	Thr	Gly	His	Gln	Leu	Leu	Cys	Asp	Lys	Cys	Ala	Pro	Gly	Thr
			35				40					45			
Tyr	Leu	Lys	Gln	His	Cys	Thr	Val	Arg	Arg	Lys	Thr	Leu	Cys	Val	Pro
						55					60				
Cys	Pro	Asp	His	Ser	Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys
					70					75					80
Val	Tyr	Cys	Ser	Pro	Val	Cys	Lys	Glu	Leu	Gln	Ser	Val	Lys	Gln	Glu
				85					90					95	
Cys	Asn	Arg	Thr	His	Asn	Arg	Val	Cys	Glu	Cys	Glu	Glu	Gly	Arg	Tyr
			100				105						110		
Leu	Glu	Ile	Glu	Phe	Cys	Leu	Lys	His	Arg	Ser	Cys	Pro	Pro	Gly	Ser
			115				120					125			
Gly	Val	Val	Gln	Ala	Gly	Thr	Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Lys
			130				135				140				
Cys	Pro	Asp	Gly	Phe	Phe	Ser	Gly	Glu	Thr	Ser	Ser	Lys	Ala	Pro	Cys
				150						155					160
Ile	Lys	His	Thr	Asn	Cys	Ser	Thr	Phe	Gly	Leu	Leu	Leu	Ile	Gln	Lys
				165					170					175	
Gly	Asn	Ala	Thr	His	Asp	Asn	Val	Cys	Ser	Gly	Asn	Arg	Glu	Ala	Thr
			180					185					190		
Gln	Lys	Cys	Gly	Ile	Asp	Val	Thr	Leu	Cys	Glu	Glu	Ala	Phe	Phe	Arg
			195				200					205			
Phe	Ala	Val	Pro	Thr	Lys	Ile	Ile	Pro	Asn	Trp	Leu	Ser	Val	Leu	Val
					215						220				

FIG. 2B

Asp	Ser	Leu	Pro	Gly	Thr	Lys	Val	Asn	Ala	Glu	Ser	Val	Glu	Arg	Ile	225	230	235	240
Lys	Arg	Arg	His	Ser	Ser	Gln	Glu	Gln	Thr	Phe	Gln	Leu	Leu	Lys	Leu	245	250	255	
Trp	Lys	His	Gln	Asn	Arg	Asp	Gln	Glu	Met	Val	Lys	Lys	Ile	Ile	Gln	260	265	270	
Asp	Ile	Asp	Leu	Cys	Glu	Ser	Ser	Val	Gln	Arg	His	Leu	Gly	His	Ser	275	280	285	
Asn	Leu	Thr	Thr	Glu	Gln	Leu	Leu	Ala	Leu	Met	Glu	Ser	Leu	Pro	Gly	290	295	300	
Lys	Lys	Ile	Ser	Pro	Glu	Glu	Ile	Glu	Arg	Thr	Arg	Lys	Thr	Cys	Lys	305	310	315	320
Ser	Ser	Glu	Gln	Leu	Leu	Lys	Leu	Leu	Ser	Leu	Trp	Arg	Ile	Lys	Asn	325	330	335	
Gly	Asp	Gln	Asp	Thr	Leu	Lys	Gly	Leu	Met	Tyr	Ala	Leu	Lys	His	Leu	340	345	350	
Lys	Thr	Ser	His	Phe	Pro	Lys	Thr	Val	Thr	His	Ser	Leu	Arg	Lys	Thr	355	360	365	
Met	Arg	Phe	Leu	His	Ser	Phe	Thr	Met	Tyr	Arg	Leu	Tyr	Gln	Lys	Leu	370	375	380	
Phe	Leu	Glu	Met	Ile	Gly	Asn	Gln	Val	Gln	Ser	Val	Lys	Ile	Ser	Cys	385	390	395	400
Leu																			

FIG. 3A

Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His
 1 5 10 15
 Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His
 20 25 30
 Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr
 35 40 45
 Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro
 50 55 60
 Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His
 65 70 75 80
 Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe
 85 90 95
 Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala
 100 105 110
 Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe
 115 120 125
 Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn
 130 135 140
 Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His
 145 150 155 160
 Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Ala Ala Ala
 165 170 175
 Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala
 180 185 190
 Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
 195 200 205
 Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
 210 215 220

FIG. 3B

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
 225 230 235 240
 Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
 245 250 255
 Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
 260 265 270
 Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
 275 280 285
 Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
 290 295 300
 Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr
 305 310 315 320
 Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
 325 330 335
 Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
 340 345 350
 Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
 355 360 365
 Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
 370 375 380
 Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
 385 390 395 400
 Ser Leu Ser Leu Ser Pro Gly
 405

FIG. 4A

Glu	Thr	Phe	Pro	Pro	Lys	Tyr	Leu	His	Tyr	Asp	Glu	Glu	Thr	Ser	His	1	5	10	15
Gln	Leu	Leu	Cys	Asp	Lys	Cys	Pro	Pro	Gly	Thr	Tyr	Leu	Lys	Gln	His	20	25	30	
Cys	Thr	Ala	Lys	Trp	Lys	Thr	Val	Cys	Ala	Pro	Cys	Pro	Asp	His	Tyr	35	40	45	
Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys	Leu	Tyr	Cys	Ser	Pro	50	55	60	
Val	Cys	Lys	Glu	Leu	Gln	Tyr	Val	Lys	Gln	Glu	Cys	Asn	Arg	Thr	His	65	70	75	80
Asn	Arg	Val	Cys	Glu	Cys	Lys	Glu	Gly	Arg	Tyr	Leu	Glu	Ile	Glu	Phe	85	90	95	
Cys	Leu	Lys	His	Arg	Ser	Cys	Pro	Pro	Gly	Phe	Gly	Val	Val	Gln	Ala	100	105	110	
Gly	Thr	Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Arg	Cys	Pro	Asp	Gly	Phe	115	120	125	
Phe	Ser	Asn	Glu	Thr	Ser	Ser	Lys	Ala	Pro	Cys	Arg	Lys	His	Thr	Asn	130	135	140	
Cys	Ser	Val	Phe	Gly	Leu	Leu	Leu	Thr	Gln	Lys	Gly	Asn	Ala	Thr	His	145	150	155	160
Asp	Asn	Ile	Cys	Ser	Gly	Asn	Ser	Glu	Ser	Thr	Gln	Lys	Cys	Gly	Ile	165	170	175	
Asp	Val	Thr	Ala	Ala	Ala	Glu	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	180	185	190	
Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	195	200	205	
Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	210	215	220	
Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	225	230	235	240

SUBSTITUTE SHEET (RULE 26)

FIG. 4B

Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
 245 250 255
 Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
 260 265 270
 Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
 275 280 285
 Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
 290 295 300
 Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
 305 310 315 320
 Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
 325 330 335
 Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
 340 345 350
 Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
 355 360 365
 Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
 370 375 380
 Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
 385 390 395 400
 Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
 405 410

FIG. 5A

Glu	Thr	Phe	Pro	Pro	Lys	Tyr	Leu	His	Tyr	Asp	Glu	Glu	Thr	Ser	His	1	5	10	15
Gln	Leu	Leu	Cys	Asp	Lys	Cys	Pro	Pro	Gly	Thr	Tyr	Leu	Lys	Gln	His	20	25	30	
Cys	Thr	Ala	Lys	Trp	Lys	Thr	Val	Cys	Ala	Pro	Cys	Pro	Asp	His	Tyr	35	40	45	
Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys	Leu	Tyr	Cys	Ser	Pro	50	55	60	
Val	Cys	Lys	Glu	Leu	Gln	Tyr	Val	Lys	Gln	Glu	Cys	Asn	Arg	Thr	His	65	70	75	80
Asn	Arg	Val	Cys	Glu	Cys	Lys	Glu	Gly	Arg	Tyr	Leu	Glu	Ile	Glu	Phe	85	90	95	
Cys	Leu	Lys	His	Arg	Ser	Cys	Pro	Pro	Gly	Phe	Gly	Val	Val	Gln	Ala	100	105	110	
Gly	Thr	Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Arg	Cys	Pro	Asp	Gly	Phe	115	120	125	
Phe	Ser	Asn	Glu	Thr	Ser	Ser	Lys	Ala	Pro	Cys	Arg	Lys	His	Thr	Asn	130	135	140	
Cys	Ser	Val	Phe	Gly	Leu	Leu	Thr	Gln	Lys	Gly	Asn	Ala	Thr	His	145	150	155	160	
Asp	Asn	Ile	Cys	Ser	Gly	Asn	Ser	Glu	Ser	Thr	Gln	Lys	Val	Asp	Lys	165	170	175	
Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	180	185	190	
Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	195	200	205	
Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	210	215	220	
Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	225	230	235	240

SUBSTITUTE SHEET (RULE 26)

FIG. 5B

Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	245	250	255
Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	260	265	270
Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	275	280	285
Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	290	295	300
Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	305	310	315
Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu	Trp	Glu	325	330	335
Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu	340	345	350
Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	Val	Asp	Lys	355	360	365
Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu	370	375	380
Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro	Gly	385	390	395
																		400

FIG. 6A

Glu	Thr	Phe	Pro	Pro	Lys	Tyr	Leu	His	Tyr	Asp	Glu	Glu	Thr	Ser	His	1	5	10	15
Gln	Leu	Leu	Cys	Asp	Lys	Cys	Pro	Pro	Gly	Thr	Tyr	Leu	Lys	Gln	His	20	25	30	
Cys	Thr	Ala	Lys	Trp	Lys	Thr	Val	Cys	Ala	Pro	Cys	Pro	Asp	His	Tyr	35	40	45	
Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys	Leu	Tyr	Cys	Ser	Pro	50	55	60	
Val	Cys	Lys	Glu	Leu	Gln	Tyr	Val	Lys	Gln	Glu	Cys	Asn	Arg	Thr	His	65	70	75	80
Asn	Arg	Val	Cys	Glu	Cys	Lys	Glu	Gly	Arg	Tyr	Leu	Glu	Ile	Glu	Phe	85	90	95	
Cys	Leu	Lys	His	Arg	Ser	Cys	Pro	Pro	Gly	Phe	Gly	Val	Val	Gln	Ala	100	105	110	
Gly	Thr	Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Arg	Cys	Pro	Asp	Gly	Phe	115	120	125	
Phe	Ser	Asn	Glu	Thr	Ser	Ser	Lys	Ala	Pro	Cys	Arg	Lys	His	Thr	Asn	130	135	140	
Cys	Ser	Val	Phe	Gly	Leu	Leu	Leu	Thr	Gln	Lys	Gly	Asn	Ala	Thr	His	145	150	155	160
Asp	Asn	Ile	Cys	Ser	Gly	Asn	Ser	Glu	Ser	Thr	Gln	Lys	Cys	Gly	Ile	165	170	175	
Asp	Val	Thr	Val	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	180	185	190	
Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	195	200	205	
Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	210	215	220	
Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	225	230	235	240

FIG. 6B

Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	
				245					250					255		
Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	
			260					265					270			
Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	
		275					280					285				
Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	
	290					295					300					
Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	
305					310					315				320		
Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	
			325						330					335		
Ile	Ala	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	
		340						345					350			
Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	
		355					360					365				
Lys	Leu	Thr	Val	Asp	Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	
	370					375					380					
Cys	Ser	Val	Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	
385				390						395					400	
Leu	Ser	Leu	Ser	Pro	Gly											
				405												

FIG. 7A

Glu	Thr	Phe	Pro	Pro	Lys	Tyr	Leu	His	Tyr	Asp	Glu	Glu	Thr	Ser	His	1	5	10	15
Gln	Leu	Leu	Cys	Asp	Lys	Cys	Pro	Pro	Gly	Thr	Tyr	Leu	Lys	Gln	His	20	25	30	
Cys	Thr	Ala	Lys	Trp	Lys	Thr	Val	Cys	Ala	Pro	Cys	Pro	Asp	His	Tyr	35	40	45	
Tyr	Thr	Asp	Ser	Trp	His	Thr	Ser	Asp	Glu	Cys	Leu	Tyr	Cys	Ser	Pro	50	55	60	
Val	Cys	Lys	Glu	Leu	Gln	Tyr	Val	Lys	Gln	Glu	Cys	Asn	Arg	Thr	His	65	70	75	80
Asn	Arg	Val	Cys	Glu	Cys	Lys	Glu	Gly	Arg	Tyr	Leu	Glu	Ile	Glu	Phe	85	90	95	
Cys	Leu	Lys	His	Arg	Ser	Cys	Pro	Pro	Gly	Phe	Gly	Val	Val	Gln	Ala	100	105	110	
Gly	Thr	Pro	Glu	Arg	Asn	Thr	Val	Cys	Lys	Arg	Cys	Pro	Asp	Gly	Phe	115	120	125	
Phe	Ser	Asn	Glu	Thr	Ser	Ser	Lys	Ala	Pro	Cys	Arg	Lys	His	Thr	Asn	130	135	140	
Cys	Ser	Val	Phe	Gly	Leu	Leu	Leu	Thr	Gln	Lys	Gly	Asn	Ala	Thr	His	145	150	155	160
Asp	Asn	Ile	Cys	Ser	Gly	Asn	Ser	Glu	Ser	Thr	Gln	Lys	Ser	Gly	Gly	165	170	175	
Gly	Gly	Gly	Gly	Gly	Gly	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	180	185	190	
Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	195	200	205	
Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	210	215	220	
Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	225	230	235	240

SUBSTITUTE SHEET (RULE 26)

FIG. 7B

Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	245	250	255
Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	260	265	270
Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	275	280	285
Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	290	295	300
Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	305	310	315
Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	325	330	335
Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	340	345	350
Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	355	360	365
Thr	Val	Asp	Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	370	375	380
Val	Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	385	390	395
Leu	Ser	Pro	Gly															400

FIG. 8A

Met	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	1	5	10	15
Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Lys	Pro	Lys	Asp	Thr	Leu		20	25	30	
Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	35	40	45	
His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	50	55	60	
Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	65	70	75	80
Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	85	90	95	
Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	100	105	110	
Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	115	120	125	
Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	130	135	140	
Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	145	150	155	160
Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	165	170	175	
Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser	Lys	Leu	Thr	180	185	190	
Val	Asp	Lys	Ser	Arg	Trp	Gln	Gln	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	195	200	205	
Met	His	Glu	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	210	215	220	
Ser	Pro	Gly	Lys	Glu	Thr	Phe	Pro	Pro	Lys	Tyr	Leu	His	Tyr	Asp	Glu	225	230	235	240

FIG. 8B

Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr	245	250	255
Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro Cys	260	265	270
Pro Asp His Tyr Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Leu	275	280	285
Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys	290	295	300
Asn Arg Thr His Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu	305	310	315
Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe Gly	325	330	335
Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg Cys	340	345	350
Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg	355	360	365
Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly	370	375	380
Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln	385	390	395
Lys			400

FIG. 9A

FIG. 9B

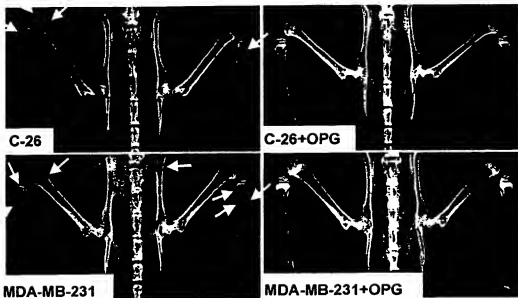


FIG. 9C

FIG. 9D

FIG. 10

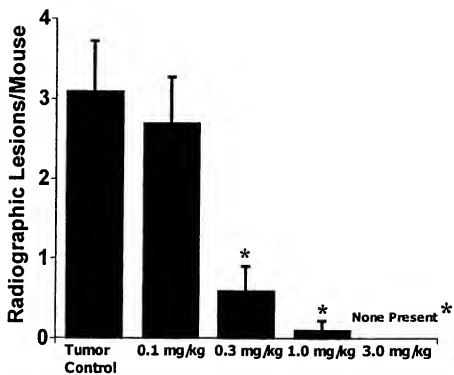


FIG. 11A

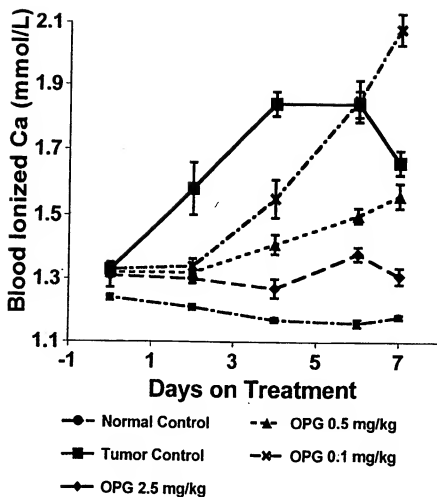
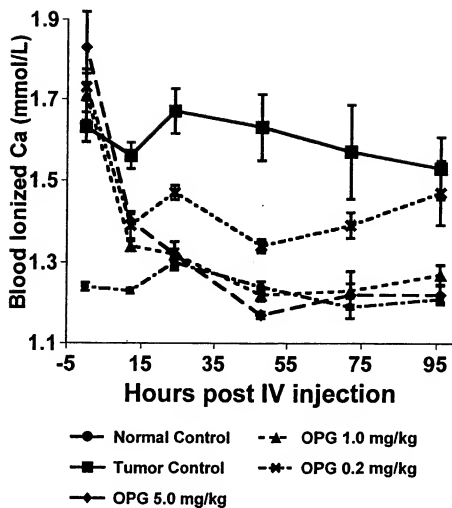


FIG. 11B



- 1 -

SEQUENCE LISTING

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TREATMENT OF CANCER AND BONE LOSS ASSOCIATED WITH
CANCER

<130> A-605

<140> 09/389,545

<141> 1999-09-03

<160> 8

<170> PatentIn Ver. 2.1

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Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
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Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
 35           40           45

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
 50           55           60

Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
 65           70           75           80

Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
      85           90           95

Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
 100           105           110

Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
 115           120           125

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr
 130           135           140

Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
 145           150           155           160

Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
      165           170           175

Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
 180           185           190

Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
 195           200           205

```

- 2 -

Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
 210 215 220
 Ser Leu Ser Leu Ser Pro Gly Lys
 225 230
 <210> 2
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 <212> FRT
 <213> Human
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 Met Asn Lys Trp Leu Cys Cys Ala Leu Leu Val Leu Leu Asp Ile Ile
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 Glu Trp Thr Thr Gln Glu Thr Leu Pro Pro Lys Tyr Leu His Tyr Asp
 20 25 30
 Pro Glu Thr Gly His Gln Leu Leu Cys Asp Lys Cys Ala Pro Gly Thr
 35 40 45
 Tyr Leu Lys Gln His Cys Thr Val Arg Arg Lys Thr Leu Cys Val Pro
 50 55 60
 Cys Pro Asp His Ser Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
 65 70 75 80
 Val Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Ser Val Lys Gln Glu
 85 90 95
 Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Glu Glu Gly Arg Tyr
 100 105 110
 Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Gly Ser
 115 120 125
 Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Lys
 130 135 140
 Cys Pro Asp Gly Phe Phe Ser Gly Glu Thr Ser Ser Lys Ala Pro Cys
 145 150 155 160
 Ile Lys His Thr Asn Cys Ser Thr Phe Gly Leu Leu Leu Ile Gln Lys
 165 170 175
 Gly Asn Ala Thr His Asp Asn Val Cys Ser Gly Asn Arg Glu Ala Thr
 180 185 190
 Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg
 195 200 205
 Phe Ala Val Pro Thr Lys Ile Ile Pro Asn Trp Leu Ser Val Leu Val
 210 215 220
 Asp Ser Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile
 225 230 235 240
 Lys Arg Arg His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu
 245 250 255

- 3 -

Trp Lys His Gln Asn Arg Asp Gln Glu Met Val Lys Lys Ile Ile Gln
 260 265 270
 Asp Ile Asp Leu Cys Glu Ser Ser Val Gln Arg His Leu Gly His Ser
 275 280 285
 Asn Leu Thr Thr Glu Gln Leu Leu Ala Leu Met Glu Ser Leu Pro Gly
 290 295 300
 Lys Lys Ile Ser Pro Glu Glu Ile Glu Arg Thr Arg Lys Thr Cys Lys
 305 310 315 320
 Ser Ser Glu Gln Leu Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn
 325 330 335
 Gly Asp Gln Asp Thr Leu Lys Gly Leu Met Tyr Ala Leu Lys His Leu
 340 345 350
 Lys Thr Ser His Phe Pro Lys Thr Val Thr His Ser Leu Arg Lys Thr
 355 360 365
 Met Arg Phe Leu His Ser Phe Thr Met Tyr Arg Leu Tyr Gln Lys Leu
 370 375 380
 Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys
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 Leu

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 Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr
 35 40 45
 Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro
 50 55 60
 Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His
 65 70 75 80
 Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe
 85 90 95
 Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala
 100 105 110
 Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe
 115 120 125

- 4 -

Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn
 130 135 140
 Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His
 145 150 155 160
 Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Ala Ala Ala
 165 170 175
 Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala
 180 185 190
 Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro
 195 200 205
 Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
 210 215 220
 Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val
 225 230 235 240
 Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln
 245 250 255
 Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln
 260 265 270
 Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala
 275 280 285
 Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
 290 295 300
 Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr
 305 310 315 320
 Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
 325 330 335
 Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
 340 345 350
 Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
 355 360 365
 Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
 370 375 380
 Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
 385 390 395 400
 Ser Leu Ser Leu Ser Pro Gly
 405

<210> 4
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 <212> PRT
 <213> Human

- 5 -

<400> 4

Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu Glu Thr Ser His
 1 5 10 15
 Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His
 20 25 30
 Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr
 35 40 45
 Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro
 50 55 60
 Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His
 65 70 75 80
 Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe
 85 90 95
 Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala
 100 105 110
 Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe
 115 120 125
 Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn
 130 135 140
 Cys Ser Val Phe Gly Leu Leu Thr Gln Lys Gly Asn Ala Thr His
 145 150 155 160
 Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys Gly Ile
 165 170 175
 Asp Val Thr Ala Ala Glu Pro Lys Ser Cys Asp Lys Thr His Thr
 180 185 190
 Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
 195 200 205
 Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
 210 215 220
 Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
 225 230 235 240
 Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
 245 250 255
 Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
 260 265 270
 Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
 275 280 285
 Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
 290 295 300
 Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
 305 310 315 320

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Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
 325 330 335
 Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
 340 345 350
 Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
 355 360 365
 Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
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 35 40 45
 Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro
 50 55 60
 Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His
 65 70 75 80
 Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe
 85 90 95
 Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala
 100 105 110
 Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe
 115 120 125
 Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn
 130 135 140
 Cys Ser Val Phe Gly Leu Leu Thr Gln Lys Gly Asn Ala Thr His
 145 150 155 160
 Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Val Asp Lys
 165 170 175
 Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
 180 185 190

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser
 195 200 205
 Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp
 210 215 220
 Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn
 225 230 235 240
 Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val
 245 250 255
 Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu
 260 265 270
 Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys
 275 280 285
 Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr
 290 295 300
 Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr
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 Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu
 325 330 335
 Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
 340 345 350
 Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys
 355 360 365
 Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu
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 Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His
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 Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr
 35 40 45
 Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro
 50 55 60

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Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His
 65 70 75 80
 Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe
 85 90 95
 Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala
 100 105 110
 Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe
 115 120 125
 Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn
 130 135 140
 Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His
 145 150 155 160
 Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Cys Gly Ile
 165 170 175
 Asp Val Thr Val Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
 180 185 190
 Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
 195 200 205
 Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
 210 215 220
 Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
 225 230 235 240
 Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
 245 250 255
 Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
 260 265 270
 Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
 275 280 285
 Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
 290 295 300
 Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
 305 310 315 320
 Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
 325 330 335
 Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
 340 345 350
 Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
 355 360 365
 Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
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Leu Ser Leu Ser Pro Gly
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Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr Leu Lys Gln His
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Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro Cys Pro Asp His Tyr
35 40 45

Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Leu Tyr Cys Ser Pro
50 55 60

Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys Asn Arg Thr His
65 70 75 80

Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu Glu Ile Glu Phe
85 90 95

Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe Gly Val Val Gln Ala
100 105 110

Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg Cys Pro Asp Gly Phe
115 120 125

Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg Lys His Thr Asn
130 135 140

Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly Asn Ala Thr His
145 150 155 160

Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln Lys Ser Gly Gly
165 170 175

Gly Gly Gly Gly Gly Gly Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu
180 185 190

Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr
195 200 205

Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val
210 215 220

Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
225 230 235 240

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
245 250 255

Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu

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260 265 270
 Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala
 275 280 285
 Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro
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 Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln
 305 310 315 320
 Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala
 325 330 335
 Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr
 340 345 350
 Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu
 355 360 365
 Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser
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 35 40 45
 His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
 50 55 60
 Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
 65 70 75 80
 Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
 85 90 95
 Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
 100 105 110
 Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
 115 120 125
 Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val

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130 135 140
 Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
 145 150 155 160
 Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
 165 170 175
 Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
 180 185 190
 Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
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 Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
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 Ser Pro Gly Lys Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp Glu
 225 230 235 240
 Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr Tyr
 245 250 255
 Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro Cys
 260 265 270
 Pro Asp His Tyr Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys Leu
 275 280 285
 Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu Cys
 290 295 300
 Asn Arg Thr His Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr Leu
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 Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe Gly
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 Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg Cys
 340 345 350
 Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys Arg
 355 360 365
 Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys Gly
 370 375 380
 Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr Gln
 385 390 395 400
 Lys

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